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Dissecting brain tumor growth and metastasis in vitro and ex vivo

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ABSTRACT

Local infiltration and distal dissemination of tumor cells hamper efficacy of current treatments against central nervous system (CNS) tumors and greatly influence mortality and therapy-induced long-term morbidity in survivors. A number of *in vitro* and *ex vivo* assay systems have been established to better understand the infiltration and metastatic processes, to search for molecules that specifically block tumor cell infiltration and metastatic dissemination and to pre-clinically evaluate their efficaciousness. These systems allow analytical testing of tumor cell viability and motile and invasive capabilities in simplified and well-controlled environments. However, the urgent need for novel anti-metastatic therapies has provided an incentive for the further development of not only classical *in vitro* methods but also of novel, physiologically more relevant assay systems including organotypic brain slice culture. In this review, using publicly available peer-reviewed primary research and review articles, we provide an overview of a selection of *in vitro* and *ex vivo* techniques widely used to study growth and dissemination of primary metastatic brain tumors. Furthermore, we discuss how our steadily increasing knowledge of tumor biology and the tumor microenvironment could be integrated to improve current research methods for metastatic brain tumors. We believe that such rationally improved methods will ultimately increase our understanding of the biology of brain tumors and facilitate the development of more efficacious anti-metastatic treatments.

Key words: Primary brain tumor; metastasis; *in vitro* model system; cell migration; organotypic brain slice culture

INTRODUCTION

Impressive achievements in genomic and epigenomic analyses of tumor tissues and individual tumor cells have revolutionized our understanding of primary brain tumors. Alterations detected on the genome or transcriptome level in large patient cohorts in combination with our increasing understanding of epigenetic gene regulation have

disentangled apparently identical brain tumors as related but functionally different tumor entities.^[1-5] Within such single tumor entities, alterations detected in their respective metastases suggested potential driver mechanisms of tumor progression.^[6] This considerably more complex image we currently have is instrumental to better understand the highly heterogeneous nature of the tumor tissue itself and of the host environment interacting with it and shaping some of

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its spatial, functional and morphological manifestations. However, in order to translate this still growing knowledge into clinical applications targeting the tumor phenotype, sophisticated model systems are necessary to explore and validate potential interference strategies under physiologically relevant conditions. In addition, functional genomics and cell-based molecular analyses are indispensable in many cases to clarify whether mutated or amplified genes are necessarily contributory to an altered proteome and causative for the cancerous phenotype. Moreover, the current wealth of genomic and transcriptomic data is insufficient on its own to isolate specific signaling networks driving tumor progression from a benign lesion to a disseminated cancer. Hence, to tackle the complexity of the metastatic process it is necessary to dissect it into individual steps that can be addressed with rationally adapted model systems. In this review we focus on *in vitro* and *ex vivo* primary brain tumor model systems and discuss how they can be improved and used to develop the molecular understanding necessary for designing novel anti-metastatic therapies. While none of these model systems on its own will suffice to tackle such a complex disease as cancer, they can effectively guide our search for efficacious and less toxic therapies and instruct the design of appropriate *in vivo* studies.

THE MACHINERY: ALTERED CYTOSKELETON DYNAMICS AND CELL MOTILITY DRIVE CANCER DISSEMINATION

Dissemination of tumor cells from the primary tumor causes healthy tissue infiltration and metastatic disease, and

it hampers the efficacy of current cancer treatments. It is triggered by the transient or permanent induction of motility and invasiveness in the tumor cells. An essential prerequisite for primary brain tumor cell migration and invasion is the remodeling of the actin and tubulin cytoskeletons,^[7-9] which not only provide force, traction and rigidity but also scaffold signaling complexes in a spatially controlled manner.^[10-12] Hence, blocking motility and invasiveness by targeting pro-migratory cytoskeleton dynamics in tumor cells could prevent local tumor cell invasion, further dissemination from proximal metastases and the evolution towards a more aggressive phenotype. In a seminal review by Giese *et al.*,^[13] the dichotomy of migration and proliferation in gliomas was recognized as the consequence of antagonistic cell regulation. Consequently, the authors concluded that an approach to influence the underlying mechanisms could be the basis of novel anti-invasive therapy strategies. A computational modeling study predicts that even a small increase in the motile capability of tumor cells, and the consequent short-range dissemination, increases net tumor growth and resistance to targeted therapy^[14] [Figure 1]. Indeed, targeting tumor cell motility and invasiveness as a strategy against metastasis is an emerging theme in cancer research,^[15-17] and the pro-migratory phenotype in tumor cells has been addressed in the past by a number of approaches that impair cell autonomous migration, cell-cell communication, cell-cell or cell-matrix interaction (^[15] and references therein). This research led to the development of a number of clinical trial studies for solid tumors with approaches inhibiting various components of the aforementioned pro-migratory determinants.^[15]

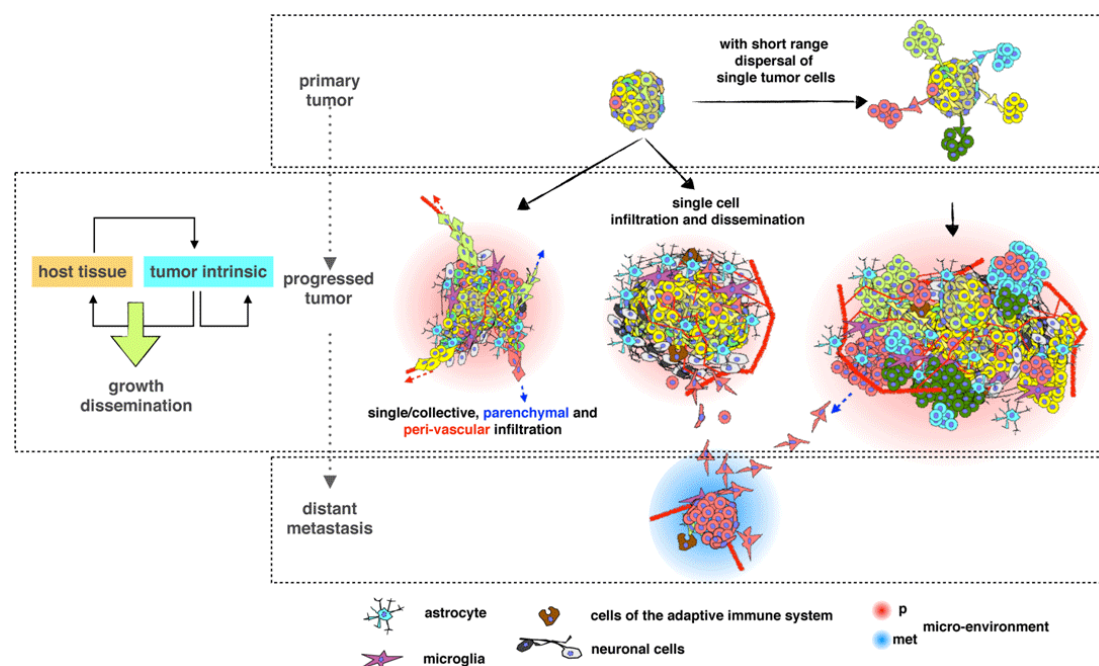


Figure 1: Model of growth, progression and dissemination of primary brain tumors. The progression of primary brain tumors from a small neoplastic lesion to a metastasizing tumor through growth and dissemination of tumor cells is schematically visualized. The mode of tumor cell growth and dissemination varies between different tumors and involves random or guided, single or collective dissemination of tumor cells. The model depicting low range dissemination at early stages and the consequent increased net tumor growth is according to Waclaw *et al.*^[14]

Despite of this, most current treatments including those against primary brain tumors still focus primarily on targeting growth and survival of the tumor cells. This lack of adequate anti-dissemination therapies is due in part to the complexity of the cell migration process itself and the redundancy of the signaling that controls its mechanics.

Additionally, tumor cells exploit mechanisms that normally direct physiological movements. However, the addition of tumor cells to druggable pathways and our increasing understanding of cell mechanics and its control offer room for therapeutic interventions targeting tumor cell dissemination specifically.

The soil: the microenvironment in the brain

To address the impact of the tumor microenvironment on tumor growth and progression *in vitro*, we need (a) to better understand the intricate interaction between a growing neoplasm and its cellular, biophysical and chemical environment and (b) to continuously implement this increasing knowledge for advancing our model systems to mimic the micro environmental parameters better. The following paragraph will briefly discuss some relevant aspects of the still poorly understood interaction between the cells of primary brain tumors and their cellular host environment.

Biophysical properties of the brain microenvironment

Mammalian cells are sensitive to biophysical and chemical signals emanating from the surrounding matrix environment, the extracellular matrix (ECM), which can influence their behavior.^[18-20] Depending on the tissue, composition and stiffness of the ECM differs markedly.^[21] The stiffness or rigidity of a material such as a meshwork of collagen I fibers, describes its resistance to deformation. It depends on the elastic modulus (or compliance) of its constituting material e.g. fibrillar polymers of the protein collagen, which describes the ability to resist a distorting influence and to return to its original size and shape when the influence is removed. Thus, the stiffness of the ECM depends on its components and their elastic modulus. As the parenchyma of the brain is mostly devoid of fibers with a high elastic modulus such as collagen or fibronectin fibrils, its stiffness is very low compared to the ECMs in other tissues of the human body.^[22] Conversely, the leptomeninges in the subarachnoid space, to where metastatic medulloblastoma tumors preferentially spread,^[23] are connected by a network of collagen-rich trabeculae,^[23,24] which likely is much stiffer than the parenchyma.

The basic constituents of the brain ECM are glycosaminoglycans with their most prominent member hyaluronan (Hyaluronic acid, HA), link proteins, lecticans and tenascins.^[25] HA acts as a backbone for the assembly of a relatively loose and flexible meshwork. The distribution and composition of these ECM components in the developing rodent brain is changing during embryonal and postnatal phases and reaches a mature stage at postnatal

day 20.^[25] However, disease-associated remodeling of the CNS ECM has been observed after injury,^[25-28] suggesting that growing primary neoplasms in the brain may also alter the surrounding ECM. Relatively little change in the expression levels of a small set of proteins in normal brain tissue and in brain tissue surrounding invasive glioblastoma was observed in a recent study,^[29] except for Tenascin-R and CD168, which were both up-regulated. Matrix stiffness regulates proliferation and motility of Glioblastoma multiforme (GBM) cells^[30] and the increase of ECM stiffness through fiber crosslinking by the product of the LOX gene causes their enhanced integrin-dependent invasion.^[31] The specific impact of matrix stiffness on cell migration was investigated in glioma and found to decrease motility in agarose-stiffened collagen gels^[19] and to increase motility in matrigel.^[32] This somewhat conflicting result may be explained by the receptors sensing the matrix environment and their underlying signaling, which markedly influence the migratory outcome. Hence, the impact of matrix stiffness on the migratory behavior should always be investigated in the context of the cognate receptors. Whether matrix stiffness could exert a selective pressure on brain tumor cells contributing to the altered genetic landscapes is still poorly understood. One potential sensor and transducer of matrix stiffness in brain tumors is the HA receptor CD44, which was identified in GBM to facilitate invasiveness in stiff matrices.^[33]

Chemical properties of the brain microenvironment

Analogous to solid tumors outside the CNS, where parallels between the inflammatory response in wounds and the host tissue response to growing neoplasms has been noted,^[34] remarkable similarities in brain tissue response after injury and in the vicinity of brain tumors exist.^[35] Tissue response is driven initially by a local repertoire of innate and adaptive immune cells that is subsequently supported by infiltrating cells of the adaptive immune system. In the brain, an immune privileged site of the human body, tissue response is driven by microglia/macrophages and astrocytes. Microglia are involved in first-line innate immunity in response to brain injury, when they convert to an active proliferating, migrating and phagocytic phenotype.^[36] Microglia and macrophages accumulate in and around glioma to which they are suspected to be attracted by glioma-secreted chemo attractants such as monocyte chemoattractant protein-3 (MCP-3), colony-stimulating factor 1 (CSF-1), granulocyte-colony stimulatory factor (G-CSF), and hepatocyte growth factor/scatter factor.^[37] Besides direct stimulatory functions through secretion of growth factors or proteolytic enzymes, glioma infiltrating macrophages were also found to contribute to tumor vascularization and net tumor growth.^[38] Surprisingly, however, malignancy or primary cranial origin did not seem to determine immune cell infiltration as no significant difference in immune cell distribution was observed between different primary or secondary brain malignancies (Glioma, PNET/Medulloblastoma,

adenocarcinoma, melanoma meningioma).^[39] A more recent study correlating inflammatory gene expression with the molecular subgroup of medulloblastoma, revealed significantly increased immune cell infiltration of tumor associated macrophages and other immune cells in the SHH subgroup,^[40] suggesting a potential therapeutic relevance of immune cell targeting specifically for this subgroup.

Microglia are outnumbered by astrocytes, which account for nearly half of all cells resident in the brain. Astrocytes respond to brain injury and tumor growth in a process named reactive gliosis. On the one hand, reactive gliosis and the associated secretion of growth factors and cytokines help repairing injury in the CNS.^[41] On the other hand, the astrocytic response in the tumor microenvironment also contributes to disease progression. Of note in this context is the capability of U87MG glioblastoma cells to induce astrocyte activation through the secretion of Receptor Activator of NF- κ B ligand (RANKL), which in turn facilitates glioblastoma invasiveness *in vivo* by releasing FGF4, FGF6, TGF- β and Hepatocyte growth factor.^[42] Consistently, co-cultured astrocytes display increased expression levels of a number of growth factors and cytokines and enhance invasiveness of glioblastoma stem-like cells.^[43] Another decisive input could stem from astrocytes activated by the neoplastic lesion and the consequent up-regulation of matricellular proteins such as secreted protein acidic and rich in cysteines (SPARC) in astrocytoma^[44] and medulloblastoma^[45] or connective tissue growth factor in glioma,^[46] which jointly with additional matricellular proteins remodel neuronal tissue during development or after brain injury.^[28] Significantly, the concept of reciprocal stimulation of tumor cells and astrocytes was recently also identified in metastatic melanoma, which elicits an inflammatory cytokine response in astrocytes that facilitates brain metastasis.^[47]

Combined, these studies emphasize the importance of

incorporating environmental parameters into experimental protocols to explore their contribution to the proteomic landscape and the functional outcomes of primary brain tumors.

CURRENT *IN VITRO* MODEL SYSTEMS TO ADDRESS FUNCTIONS OF METASTATIC PRIMARY BRAIN TUMORS

Preclinical evaluation of novel anti-metastatic therapy strategies in animal models will remain an essential step towards the development of novel therapeutics. However, cell culture models are instrumental for deciphering essential morphological and functional aspects of the biology that drives neoplastic lesions into disseminated diseases. They also provide essential insights for designing appropriate animal models and help elucidating the causes that may underlie controversial outcomes of *in vivo* studies. Although a general trend towards 3D model systems can be noted, a majority of experiments in tumor-related research are still conducted in 2D settings. For a general, in depth description and comparison of 2D versus 3D culture systems, the reader is referred to Zimmermann *et al.*^[48] who emphasized the need of higher throughput approaches to understand cell dissemination capabilities on one hand and the role of the microenvironment on the other hand.

The “ideal” *in vitro* cell culture model should mimic one or several of the following characteristics of the *in vivo* tumor: proliferative capabilities and morphology of the tumor cells, cellular and phenotypic heterogeneity, a dynamic tumor microenvironment and the drug response profile. A series of excellent reviews have recently described in depth the use of 3D tissue culture model systems in pathophysiology^[49] and high-throughput drug candidate toxicity analysis,^[50] to identify tumor-specific signaling pathways and biomarkers,^[51] and to determine growth determinants for drug target discovery.^[52] These reviews delineate what parameters contribute to a disease representing, efficient

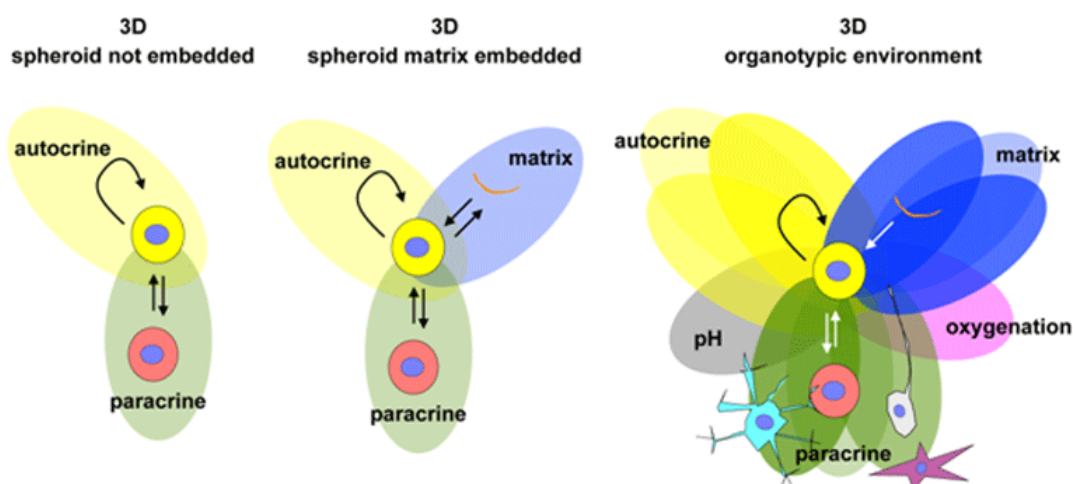


Figure 2: Tumor cell growth, survival and dissemination are governed by extrinsic and intrinsic parameters. Tumor cells are under the spheres of influence of intrinsic and extrinsic parameters. Colored ovals represent various degrees and manifestation patterns of such parameters, which dramatically increase in number and complexity in the organotypic environment

Table 1: Studies using 3D primary brain tumor model systems

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
U87MG	Glioblastoma multiforme (GBM)	2D, 3D neurospheres	Analysis of cell growth in neurospheres, wound healing after DDX6 or PHLDB1 knock-down	None	50 % reduction in neurosphere formation and migration	[83]
U-251MG, U-343MG, LN-229	Glioma	2D, 3D spheroid culture	Analysis of stable over-expression of wt and mutant proteins under different oxygenation.	None	Mutant IDH1 causes reduced cell migration and differences in growth properties in 3D spheroid cultures.	[84]
U87	GBM	2D, 3D single cell embedding	Spheroid in soft agar, 2D culture, analysis of Glioma co-culture with MSCs expressing suicide gene. Rotary cell culture to generate large cell aggregates, growth on top of matrigel.	Soft agar	Stem cell-mediated anti-tumor effect. Increased IC50 under 3D culture conditions.	[85]
KNS42, U87, Res196, T7/11, GB-1	Pediatric and adult GBM and ependymoma, pediatric mixed glial tumor	3D, long-term culture of large cellular aggregates		None	Angiogenic change and endothelial marker expression in GBM aggregates	[86]
SHSY5Y, T98G, U138MG	GBM and neuroblastoma	3D, spheroid culture	Exposure to Doxorubicin, Etoposide and Vincristine, analysis by electrochemical impedance spectroscopy.	None	Cytotoxic effect measured in 2D cannot be extrapolated to 3D. 3D cultures can also display higher sensitivity to chemotherapeutics.	[58]
U67-MG	GBM	2D	2D wound healing, tubulogenesis assay on matrigel after exposure to rapamycin or hypoxia.	None	Tube structure formation	[87]
Rat C6, NSCs adult hippocampal	Glioma	3D, spheroid culture	Comparison of different 3D tissue clearing protocols.	None	Validation of tissue clearing protocols of high resolution imaging of spheroid culture. Increased HLA-E expression in 3D culture and increased resistance to NK-mediated cytotoxicity.	[88]
U251	Glioma	2D and 3D rotary cell culture system	Proteomic comparison of 2D and 3D cell culture.	None		[89]
DBTRG, T98, U87, A172, 8MGBA, 42MGBA, DKMG, GAMG, GMS10, GSCs	GBM	2D, 3D, single cell embedding	Cytotoxicity assays using collagen I and collagen I-HA matrices in combination with receptor tyrosine kinase inhibitors.	Bovine skin collagen I, Collagen I-HA mixtures	Collagen-based 3D matrix reduces sensitivity of GSCs to receptor tyrosine kinase inhibitors.	[60]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
U87-MG, primary MB samples	GBM	2D, 3D culture	Comparison of 2D and 3D cultures for growth and viability after irradiation or treatment with TMZ, cisplatin or carmustin.	ExtracelTM (Polyethylene-based hydrogel with HA and gelatin).	3D cell culture is better morphological correlate to <i>in vivo</i> tumor, 3D grown GBM moderately less sensitive to irradiation.	[90]
U118-MG	GBM	3D culture, cells grown on rigid matrix	Evaluation of growth and stem cell properties	Porous chitosan-HA	Porous chitosan-HA increases growth and causes up-regulation of stem-cell markers	[63]
Patient-derived tumor material	GBM	3D, single cells	Embedding in hydrogels, morphology and cell migration analysis, single cell tracking for motility.	Hydrogels made of collagen I, III, or collagen-HA mixtures	HA causes rounded morphology and reduces motility of matrix-embedded cells.	[62]
U87-MG, U87+EGFR	Glioma	3D, single cells	Analysis of growth, metabolic activity and HIF-1 VEGF, MMP-2, MMP-9 and Fibronectin production.	GelMA or PEG4A hydrogels supplemented with methacrylated HA (HAMA) at increasing concentrations	Increasing HAMA concentrations cause up-regulation of fibronectin, VEGF and HIF-1.	[64]
A-172	Glioma	3D, single cells, microfluidic chip	Analysis of single cell viability, F-actin size and cellular orientation in embedded cells under flow and VEGF in microfluidic chip.	Acrylated HA cross-linked with MMP-sensitive or RGD peptides	F-actin reorganization and re-orientation of cells in response to flow and VEGF.	[91]
OSU-2	GBM	3D, single cells	Evaluation of matrix stiffness impact on tumor cell morphology and migratory/invasive capabilities.	Matrigel with varying stiffness	Increased matrix stiffness causes increased invasive motility.	[32]
M059K, HepG2, CYP3A	GBM, Hepatoblastoma	3D, micro-scale perfusion system	Evaluation of liver cell metabolism on cytotoxic effect of IFO and TMZ.	Polylactic acid scaffold	TMZ showed much lower cytotoxicity against GBM cells in 3D than in 2D. IFO effect dependent on metabolic activity of cytochrome P450 in hepatocytes.	[68]
U251MG, LN229 and U87MG	GBM	3D, organotypic slice culture	Evaluation of Rho-family GTPase activation during GBM invasion in brain slice and 3D matrigel culture. Use of Rho-family GTPase fluorescent protein sensors.	Matrigel, slice culture	Perivascular and intraparenchymal invasion is associated with increased Rac and Cdc42 and reduce Rho GTPase activity.	[53]
U87, U251HF, SNB19, LN2308, LN229	Glioma	2D, 3D	Comparison of protein expression in cells grown under 2D or 3D conditions and in different oxygenation.	AlgiMatrix	Differential expression of invasion, survival and hypoxia driver proteins between 2D and 3D. Effect of 3D growth dominates oxygenation.	[92]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
LN18, GL15, U87, A172	GBM	2D, 3D	Seeding of GBM cells on brain Hi-spots and exposure to anti-proliferative drugs Ara-C, Taxol and TMZ	Brain Hi-Spots	Increased anti-proliferative effect of TMZ on GBM cells maintained on Hi-spots.	[65]
C6 rat, U-87 MG, U-118 MG	Glioma	2D, 3D, Matrigel and chitosan-alginate scaffolds	Comparison of growth and morphology and secretion of VEGF, MMP2, fibronectin and Laminin between cells grown in 2D, in matrigel or on chitosan-alginate scaffolds.	Matrigel, Chitosan-alginate scaffolds	Growth on chitosan-alginate scaffolds reduces growth but increases secretion of VEGF, MMP2, fibronectin and Laminin.	[93]
LN18, F98, F98EGFR- vIII, C6 rat, U-87 MG,	Glioma	2D, 3D spheroid culture, transwell migration	Evaluation of SHA impact on cell growth, collagen I invasion and mRNA expression of genes relevant for cell-cell and cell-matrix interaction.	Collagen I	SAHA treatment causes reduction of invasion and the reorganization of the matrix surrounding the tumor spheroids.	[94]
U178, U251	Glioma	3D transwell	Analysis of transwell invasion and migration after compound inhibition of PKC δ .	Collagen I supplemented with Tenascin C	Tenascin-C deposition triggers glioma invasion in a PKC δ -dependent manner.	[95]
U373	Glioma	3D spheroid	Analysis of growth and dissemination in increasingly stiff collagen I gels.	Collagen I-agarose	Matrix stiffness impacts on glioma cell invasiveness. High stiffness blocks invasiveness.	[19]
U251, U178	Glioma	3D, single cells	Quantification of transwell migration of cells stimulated with TNF- α , IL-1 or a combination of both.	Collagen I	Interleukin-1 beta (IL-1b) and tumor necrosis factor-alpha (TNF- α) increase glioma cell invasiveness in 3D with parallel increased MMP-2 and MMP-9.	[96]
Primary mouse G3 MB	Medulloblastoma	3D neurospheres	Neurosphere compound toxicity assays using FDA-approved drugs and ATP-sensor dye.	None	FDA-approved Pemetrexed and Gemcitabine significantly block proliferation of G3 MB.	[55]
DAOY, UW228	Medulloblastoma	2D, 3D transwell, 3D micro beads	Quantification of collagen invasion after HGF stimulation, small compound kinase inhibitor or siRNA treatment in cells seeded on Micro-beads and embedded in collagen I matrix.	Collagen I	HGF-induced c-Met activation promotes MB cell invasion through the kinase MAP4K4.	[9]
DAOY, UW228, Med PDX1712, MedPDX411, primary MB	Medulloblastoma	2D, 3D micro beads and spheroids	Quantification of collagen invasion and cell migration after growth factor stimulation using invasion counter platform for automated quantification of motile cell behavior in different environments.	Collagen I	HGF, EGF and bFGF are strong promoters of MB cell migration and invasion	[56]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
DAOY, UW228	Medulloblastoma	3D, transwell migration	Quantification of VEGF-A induced, PERK-dependent transwell migration.	Matrigel	Tumor cell-derived VEGF-A promotes medulloblastoma cell migration and invasion through VEGFR2 and enhanced by PERK.	[97]
DAOY, UW228	Medulloblastoma	3D, transwell migration	Quantification of EphB1 effect on SHH medulloblastoma transwell migration using electrical impedance measurements.	None	Knockdown of Eph-B1 causes reduction in B-1 integrin expression and in growth and migration.	[98]
DAOY	Medulloblastoma	3D, μ Lane microfluidics system	Quantitative and qualitative analysis of chemotactic response of MB cells to a gradient of EGF in a microfluidic system.	Matrigel	Matrigel invasion of MB cells towards an EGF gradient is blocked by pharmacological PI3-K inhibition.	[99]
DAOY	Medulloblastoma	3D, transwell migration, xCelligence assay	Quantitative analysis of PDGFR control of CXCR4 pro-migratory signaling in SHH MB model.	Matrigel	PDGF signaling restricts expression of negative regulator GRK6 and promotes CXCR4-Src-dependent cell migration.	[100]
DAOY, UW228-3	Medulloblastoma	3D confrontation co-culture	Quantification of repulsive action of Slit-Robo signaling during MB invasion.	Collagen I	Slit represses MB invasion in collagen gels.	[101]
DAOY	Medulloblastoma	2D/3D transwell migration	Evaluation of impact of matricellular SPAR on MB cell migration and invasion	Matrigel	SPARC suppresses migration and invasion by repressing Rho-GTPase activation and by triggering Src-dependent cytoskeleton reorganization. Highly self-renewing CD271 high, CD133 low MB cell population in the core sustains tumorigenesis.	[45]
DAOY, D283	Medulloblastoma	2D spheroid outgrowth, 3D transwell migration	Comparison of invasion and self-renewal. Analysis of higher versus lower self-renewing tumor spheres and stationary versus migrating adherent MB cells with respect to CD271 and CD133 expression.	Collagen I	Commitment to migration/invasion (metastatic phenotype) is identified by reduced CD271 and increased CD133 signature.	[102]

Overview of a selection of primary brain tumor studies that used 3D cell culture technologies. Ara-C: cytosine β -D-arabinofuranoside; CXCR4: CXC-motif-chemokine receptor 4; PERK: pancreatic endoplasmic reticulum kinase; EGF: epidermal growth factor; GBM: glioblastoma multiforme; GM-CSF: granulocyte-macrophage colony stimulating factor; GSCs: glioblastoma stem cells; GRK6: g-protein coupled receptor kinase 6; HA: hyaluronic acid; HAMA: methacrylated HA; HGF: hepatocyte growth factor; IDH1: isocitrate dehydrogenase 1; IFO: ifosfamide; MB: medulloblastoma; MMP: matrix metalloproteinase; PEG: polyethylene glycol; PDGFR: platelet-derived growth factor receptor; PI3-K: phosphoinositide 3'Kinase; RGD: L-arginine, glycine, and L-aspartic acid; SAHA: suberoylanilide hydroxamic acid (or vorinostat a HDACi); SPARC: secreted protein acidic and rich in cysteine; Src: rous sarcoma kinase; TMZ: temozolomide; VEGF: vasculature endothelial growth factor; 2D/3D: two dimensional/three dimensional

model system: the system should mimic biophysical and chemical properties of the tissue environment (composition and stiffness of matrix, availability of growth factors,

cytokines, metabolites) in a well controllable manner, the cells should be observable to increase output options (morphological analysis, use of fluorescent protein^[53] and dye

sensors^[54]) and it should have a high-throughput potential.

To understand the causes and consequences during pathophysiological progression from a primary neoplastic lesion in the brain towards a metastatic cancer and to pre-clinically test potential intervention strategies, we thus require model systems that mimic not only the proteomic heterogeneity of the tumor cell itself but also the reciprocal interactions between the tumor and the receiving brain tissue [Figure 2]. The following paragraph provides an overview over some recent approaches in primary brain tumor research. It highlights the difficulty to design an optimal, tumor-adapted system and emphasizes the need to further improve currently used systems.

2D and 3D model systems in primary brain tumor research

A number of articles have been published in the last few years that used *in vitro* model systems to evaluate effects of novel potential treatment strategies on growth, viability or motile behavior of primary brain tumors [Table 1]. A general consensus has been reached in that 3D cell culture model systems reflect the specifics of the *in vivo* situation better compared to 2D model systems. On the down side of this was the lack of high-throughput capability of 3D methods that hampered until a few years ago their broader use in combination with in screening approaches. A milestone in this context was the generation of spheroid cultures in 96 or even 384 well format from primary brain tumors that allowed the parallel testing or large sample sizes.^[54-56] In these studies, diagnostic dyes and fluorescent proteins were used individually or in combination for probing cellular functions on the one hand and for discriminating specific cell populations on the other hand. A general protocol describing the reproducible establishment and microscopy-based analysis of spheroid cultures using fluorescent protein quantification in high throughput was described recently.^[57] As an alternative to fluorometric read-outs, electrochemical impedance spectroscopy was used to quantify different susceptibilities of 2D versus 3D spheroid culture of glioblastoma and neuroblastoma cell lines to cytotoxic compounds^[58] and to determine the therapeutic window of these compounds. Using different combination of dyes to separate subpopulation of cells grown in co-culture combined with diagnostic flow cytometry and two-photon microscopy allowed to further refine the selective output of 3D methods.^[54] However, high-throughput capabilities and accuracy of a selected read-out has to be carefully balanced and discriminating phenotypic differences at single cell level in 3D cultures in high throughput remains a formidable challenge.

The impact of the embedding matrix on the behavior of the tumor cell

The choice of the embedding matrix is of outmost importance for 3D cultures, in particularly for primary brain tumors that encounter *in vivo* mostly brain parenchyma and collagen-rich surfaces and structures in the subarachnoid space.^[23,24,59] Hence, the biophysical and chemical properties

of the matrix should be adjusted to those in the location of growth and metastatic dissemination of the tumor under investigation. In this context, Fernandez-Fuente *et al.*^[60] investigated the impact of different environmental conditions on glioblastoma stem cells (GSCs). They found that GSCs grown in collagen-based 3D conditions were markedly less susceptible to receptor tyrosine kinase inhibition by currently available inhibitors, suggesting that oncogene addiction of tumor cells could also be bypassed by adhesion signaling.^[61] Interestingly, matrix stiffness or the addition of hyaluronic acid (HA) did not affect the sensitivity of the GSCs in this study. Primary cells from glioma patient tumor material exposed to increasing concentrations of HA responded with rounded morphology and reduced migration, suggesting that HA concentrations may affect glioma cell behavior.^[62] Consistently, addition of HA to porous chitosan scaffolds^[63] or to artificial hydrogels^[64] increased the expression of stem cell markers and VEGF and HIF-1, respectively. However, the finding that increasing matrix stiffness - by adding agarose to a collagen I matrix - blocks glioma invasiveness,^[19] suggested that stiffness alone and independent of ligand binding acted as a critical determinant for primary brain tumor cell function. An improved *in vitro* environment for brain tumor research would consist of neuronal and brain-resident interstitial cells that secrete the brain-specific ECM components into which the brain tumor cells can then be implanted. Such an environment was established from brain tissue extracts on micro filters (Hi-spots) on which GBM cell sensitivity to anti-proliferative compounds was tested.^[65] Despite its high-throughput potential, a setback of this method is the lack of control over the cellular composition in the Hi-spots and the absence of brain-specific architectural organization. A while ago, a simple but intriguing co-culture model of medulloblastoma and leptomeningeal cells was published, and it indicated paracrine, growth-promoting effects of latter that might be instrumental for studying the notoriously difficult to grow primary tumor cells *in vitro*.^[66] The ideal “organotypic environment” for primary brain tumor research was already in development in the early seventies of the last century, when the organotypic brain slice culture (OBSC) technology was established.^[67] The advantages of OBSCs are that micro environmental parameters and a relatively correct architectural organization are maintained that mimic the *in vivo* situation (see below).

Increasing complexity: system impact and single cell behavior

Neither are tumor functions disconnected from other tissues and the organs nor can the impact of tissues or organs on drug efficacy in the targeted tumor be predicted. An interesting approach to evaluate the effect of metabolic activity on cytotoxicity of compounds and chemotherapeutics *in vitro* was tested by Ma and colleagues using a 3D micro-tissue perfusion system.^[68] TMZ and IFO were perfused through hepatocytes before exposure to GBM cells and a clear impact of hepatocyte-provided cytochrome P450 on IFO activation could be shown. Analogous experimental follow-

ups are a number of organ on a chip technologies that are currently developed for assaying different disease states^[69] and testing drug effects and metabolism.^[70]

On the opposite side of the spectrum is the need to resolve the mechanisms underlying brain infiltration of single tumor cells, which necessitates approaches allowing the quantitative analysis of molecular events in individual cells. This problem was tackled for the activation status of the important Rho family GTPase's - Rho, Rac and Cdc42 - in glioma cells.^[53] Hirata *et al.*^[53] used Rho-GTPase-FRET (Förster energy resonance transfer) probes, where spatial activation of the GTPase's was monitored by a shift in fluorescence signal. Rho-family GTPase-FRET fusion protein-expressing glioma cells were orthotopically implanted in rat brains and later analyzed inside brain slice cultures derived of these brains using two-photo microscopy. This study revealed higher Rac1 and Cdc42 and lower RhoA activities in glioblastoma cells penetrating the brain parenchyma than those advancing in the perivascular regions, and suggested that different driver mechanisms could exist for single cell dispersion in glioma.

Together, these studies highlight the need for adapting the model system to the specifics of the biological context, with the consequent inclusion of biophysical or chemical components that best reflect the *in vivo* situation. Besides high-throughput screening platforms for the identification of novel pro-metastatic key players or alternative interference strategies against metastatic dissemination, we also need improved phenotype-based single cell analysis to decipher clonal differences and micro environmental impact on tumor behavior at the single cell level.

Organotypic brain slice culture (OBSC) in primary brain tumor research

A number of causal gene(s) and associated genetic mutations, molecular changes, probable targets and treatments for a variety of primary brain tumors have been identified. Despite of this, the process of dissemination, metastasis of the tumor cells from the primary site, and tumor recurrence, which is the leading cause for brain tumor related mortality in patients, remain obscure. Total removal of the primary tumor is on many occasions impossible at the microscopic level due to the insidious infiltration of the tumor cells into the surrounding brain tissue.^[71] This majorly results in therapeutic failure and urges for model systems that allow addressing brain tumor cell invasion specifically. Standard 3D *in vitro* invasion assays use ECM macromolecules that mimic the basement membrane (e.g. matrigel) as barriers to tumor invasion. These assays (described above and in table 1) although quick, reliable, commercially available and easy to perform, have several limitations. They do not take into account the unique ECM composition in the brain and thus provide artificial environments that fail to closely mimic the normal brain tissue/tumor environment. This is further emphasized by the fact that distinct types of brain tumors

localize within specific regions of the brain, highlighting the need for different microenvironments for modeling tumor growth and invasiveness. To circumvent this, mouse models have been generated for studying tumor propagation via orthotopic or subcutaneous xenografting of tumor cells. These experiments, however, are ethically controversial if inappropriately conducted, costly, labor intensive and need lengthy time periods for animal surgery and subsequent tumor development (especially for low grade tumors). These challenges and limitations highlight the need for developing a novel system wherein living brain tissue can be used as an ideal matrix for studying tumor cell growth and invasion.^[72] One such system is the organotypic culture, where cellular constituents of organs or parts of organs are allowed to regrow into or persist as organ replacements.

An excellent overview of 3D organotypic cultures has recently been provided,^[73] which describes their potentials as experimental systems to visualize cellular mechanisms that drive tissue development, to study the genetic regulation of cell behaviors in tissues and to evaluate the role of micro environmental factors in normal development and disease. One hallmark of organotypic cultures is the tissue environment mimicking the structural and functional specifics of the organ of origin. This turns them into attractive models for cancer research to explore tumor host tissue interactions and to advance therapeutic approaches.

Organotypic brain slice culture for visualization and quantification of brain tumor cell dissemination

OBSCs allow culture, maintenance and long-term survival of sections from any tissue of the CNS. Slices are mostly cultured at an air/liquid interface by either continuous rotation using the roller tube method or on a semi porous membrane using the Stoppini method.^[74] Brain tissue slice cultures maintain their normal cytoarchitecture, complex cell relationships and biochemical and electrophysiological properties. OBSCs have been widely used in the field of neurobiology for synaptogenesis, neurogenesis, myelin formation, as models for studying neurodegeneration, for neuroprotective and neurotoxic assays, *etc.*^[67] In the field of brain tumor research, they are an ideal platform to access the tumor microenvironment under intact anatomical conditions. Indeed, Jung *et al.*^[71] established a brain tumor slice model wherein they used human white matter specimens in the upper chambers of transwell culture dishes. After 24 h, control human astrocytoma cells stably expressing enhanced GFP or GFP-RHAMM (receptor for hyaluronan-mediated motility) transfected astrocytoma cells were placed in a small centrally punched-out hole in the slice. The infiltration and migratory behavior of the GFP-expressing astrocytoma cells could be easily studied using confocal laser scanning microscopy (CF-LSM) up to 30 days post implantation. The authors were able to demonstrate that different astrocytoma cell lines display different degrees of invasion and that the migration of the human astrocytoma cells could be

stimulated or, using antisense targeting strategies, specifically blocked.^[71] In an analogous study it was demonstrated that (1) the invasive behavior of the astrocytoma cells in the brain slice co-culture is not always identical to the results obtained from 2D migration studies, (2) the tumor cells spread out multidirectionally, (3) frozen human normal brain tissue can be used for the organotypic culture, (4) there were no obvious signs of necrosis, and (5) the brain cytoarchitecture and viability was preserved for at least 14 days.^[72]

Although the human origin of the biopsies used as the host tissue in these studies excludes species-specific effects in the co-culture, slices from newborn rat or mouse brains are excellent alternatives. They offer several advantages: brain regions corresponding to the *in vivo* tumor localization can be chosen, developmental stage of the brain slice can be adjusted, multiple replicas from same brain region can be generated, and the use of transgenic animals allows modification of the cellular microenvironment. Ohnishi *et al.*^[75] established OBSCs from 2-day-old neonatal rat brains, which were transferred on double-layered membranes consisting of two different membrane types and maintained at an interface between the air and the culture medium. The slices were then co-cultured with C6 glioma cells labeled with PKH2 fluorescent dye. After 2 days of co-culture, the exogenous application of the chemotactic stimulator neural cell adhesion molecule L1 triggered tumor cell migration from the upper to the bottom membrane through the brain slice.^[75] Since this study lacked CF-LSM analysis, OBSCs were subsequently performed by the slightly modified Stoppini method, which allowed quantifying glioma cell invasion using confocal microscopy.^[76] This study revealed that the migrating cells showed a strong increase in immunoreactivity for matrix metalloproteinase 2 and 9.^[76] Analogous OBSC technology was later used for mouse brain slices to quantify the invasiveness of glioma^[77] and to correlate it with histological type.^[78] Both studies used human, DiI-stained glioma biopsy tumor fragments and GFP-expressing spheroids directly implanted in the cortex of brain slices derived from 7 day old mice. This intraslice implantation system could be maintained in culture for 2 to 4 weeks. Quantification of the distance and density of the tumor cell invasion revealed that GBMs were 2-4 times more invasive than the lower grade glioma cells (LGGs). Within the different groups and grades of GBMs and LGGs, heterogeneity in terms of invasion was seen. It was also observed that the spheroids were less invasive in comparison to the directly grafted fragments. Overall using this system, Palfi *et al.*^[77,78] and de Bouard *et al.*^[77] could successfully recapitulate, monitor and quantify the invasion of single cells and the dissemination of glioma *ex vivo*. Recently, Chadwick *et al.*^[79] developed OBSCs from postnatal day 6 mice and cultured the whole brain slices on membrane inserts coated with laminin. Tumor cells (astrocytoma and medulloblastoma) were stained with Cm-DiI for monitoring, and dispensed on the center of the slice. This co-culture system remained viable for one week and effects of drug therapies on tumor cell proliferation, cell

death or changes in protein expression were successfully analyzed. Thus, Chadwick *et al.*^[79] used the OBSC system as a qualitative and quantitative assay to calculate the fold change in the number of cells during the period of slice culture. Furthermore, they investigated either the whole brain or specific regions within the brain, to assess environmental impact on primary brain tumor cell growth.

Organotypic brain slice culture to study the micro environmental impact

Malignant astrocytoma/GBM cause mortality by local tumor growth and brain invasion rather than systemic metastasis. GBM tumor cells diffusely infiltrate the brain parenchyma within and along the white matter tracts or around cerebral blood vessels,^[53] and rarely penetrate basal lamina structures at the glial limitans externa. Analogously, malignant medulloblastoma must also infiltrate cerebellar tissue for distal dissemination. Moreover, resection of MB tumors is inevitably followed by relapse if the patients are not treated with cranio-spinal radiotherapy and chemotherapy, suggesting the occurrence of local dissemination of tumor cells from the primary medulloblastoma. *In vitro* studies aiming at better understanding the local invasion process have been hampered by the lack of identification of the brain ECM macromolecules involved and the only poorly understood implication of the cellular microenvironment. *In vivo* approaches on the other hand, offer too little spatial and temporal resolution to monitor tumor-microenvironment interactions appropriately. Thus, OBSCs could provide an important platform to study the cross-talk between the tumor cells and normal cells in a physiologically relevant environment. OBSCs can be used for investigating the microenvironment and its impact on the growth and spread of primary brain tumors, and for testing the measures that could be taken to prevent or treat it effectively.^[79] Although, there is a lack of vascular supply to the tissue in the slices, capillaries do survive in these sections without any circulation.^[80] Despite of the fact that there is no blood flow and that the capillaries are not functional, it is likely that they are still capable of expressing and secreting various molecules,^[81] which could affect other cell types in the slice culture including the tumor cells. In addition, the intriguing exchange between tumor cells and astrocytes and the suspected tumor promoting functions of astrocytes^[41-43] urges for novel studies addressing the therapeutic potential of the astrocyte-tumor interaction, for which organotypic slice culture would be an ideal system.

Along with their use for monitoring tumor dissemination, OBSCs have also been used for high resolution imaging of cytoskeletal structures in living glioblastoma cells. For this, glioblastoma cells were transfected with GFP-actin and placed onto murine brain slices and spinal cord explants. Using live-cell imaging to visualize the cytoskeleton of the tumor cells, a major change in the gross morphology from a solid, two dimensional state to a three dimensional substrate was noted. This morphological change was characterized by long, dendritic-like processes that displayed regions

of ruffling activity and filopodial protrusions and by down regulation of stress-fibers.^[82]

Thus, OBSC is an excellent technology to address a wide range of topics in primary brain tumor research, ranging from growth- and dissemination-promoting signaling, to the intricate interrelations between the tumor and its surrounding host tissue to the evaluation of efficaciousness of novel targeting strategies.

FUTURE PERSPECTIVES

Main emphasis for improving current *in vitro* technologies should be given to the cellular composition and the biophysical and chemical environment conditions under which the experiment is performed. The microenvironment of the *in vivo* location of the tumor and the composition of the neuronal and interstitial cells resident in this location should guide the choice of the components. At the single cell and population levels, molecular sensors for specific cell functions should be used for probing tumor cell behavior and therapeutic efficacy. Finally, an increased output should be strived for to enable pharmacological and genetic screening approaches for drug target identification. Thus, an organotypic environment, specific read-outs and the high throughput capability will be the three pillars of future *in vitro* approaches. A great potential lies in organotypic slice culture, and when this technology is combined with state-of-the-art microscopy, it will allow to reveal fundamental aspects of local tumor cell infiltration, the interaction of neuronal and brain interstitial cell populations with the tumor cells and the evaluation of the efficaciousness of novel treatments.

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Conflicts of interest

There are no conflicts of interest.

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